INHIBITION OF MELANOGENESIS THROUGH THE DEACTIVATION OF TYROSINASE ACTIVITY IN MELANOMA CELLS BY *LABISIA PUMILA* FRACTIONS

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Dedicated to my beloved mom, my dad, my wife, Kasmah Manur, and my children, Ahmad Qaid Isyraf and Qaireen Inarah.

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ABSTRACT

Effective and safe tyrosinase inhibitors, especially those from natural sources, are desired for the application in food and whitening cosmetic products. In this research, characterization and *in-vitro* study of *L. pumila* methanol fractions have been carried out. *L. pumila* methanol fractions were prepared using solid phase extraction (SPE) and labelled as 40%, 60% and 100% methanol fractions. From characterization by total phenolic content (TPC) analysis, 40% and 100% methanol fractions were found to give the highest amount of phenolic content. The potential tyrosinase inhibition effects of *L. pumila* methanol fractions were evaluated using B16F1 melanoma cells. The cytotoxicity of the methanol fractions was evaluated using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5) diphenyltetrazolium bromide) assay. The results revealed that concentration up to 0.1% w/v of the *L. pumila* methanol fractions did not affect the cell viability. The secreted melanin had gradually decreased after the treatment with 0.025-0.1% (w/v) of *L. pumila* methanol fractions. *L. pumila* methanol fractions for both 40% and 100% methanol fractions had displayed an even stronger inhibitory effect on the secretion of melanin. The effect of melanogenesis on B16F1 indicated that both 40% and 100% methanol fractions had shown a decrease in melanin content between 0.025-0.1% (w/v) concentrations which is better than kojic acid. The melanin content for both 40% and 100% methanol fractions were 64.95 ± 1.87% and 71.91 ± 1.18%, respectively and significantly inhibited the tyrosinase activity in a dose-dependent effect of the *L. pumila* methanol fractions on the B16F1 melanoma cells. However, the 40% methanol fraction exhibited the highest inhibition up to 73.96 ± 1.58% of tyrosinase activities. Finally, the study indicates that the *L. pumila* methanol fractions have good potential as the sources of tyrosinase inhibitor.

ABSTRAK

Perencat tirosinase yang berkesan dan selamat, terutamanya dari sumber semula jadi, sangat dikehendaki bagi penggunaan di dalam produk makanan dan kosmetik pemutihan kulit. Dalam kajian ini, pencirian dan penelitian *in-vitro* bagi pecahan metanol *L. pumila* telah dikaji. Pecahan metanol *L. pumila* telah disediakan dengan menggunakan pengekstrakan fasa pepejal (SPE) dan dilabelkan sebagai 40%, 60% dan 100% pecahan metanol. Keputusan pencirian melalui analisis kandungan jumlah fenol (TPC), 40% dan 100% pecahan metanol telah memberikan jumlah tertinggi kandungan fenolik. Potensi kesan perencatan tirosinase bagi pecahan metanol *L. pumila* telah dinilai menggunakan sel-sel melanoma B16F1. Kesan sitotoksik pecahan tersebut telah dinilai dengan menggunakan ujian MTT (3-(4, 5 Dimetilthiazol-2-yl)-2,5) diphenil tetrazolium bromida). Keputusan menunjukkan bahawa pada kepekatan sehingga 0.1% w/v bagi pecahan metanol *L. pumila* tidak menjejaskan kebolehhidupan sel. Rembesan melanin secara beransur-ansur menurun selepas perawatan dengan 0.025-0.1% (w/v) pecahan metanol *L. pumila*. Pecahan metanol *L. pumila* untuk kedua-dua iaitu 40% dan 100% menunjukkan kesan perencatan yang lebih kuat pada rembesan melanin. Kesan melanogenesis pada B16F1 menunjukkan bahawa kedua-dua pecahan metanol tersebut mengakibatkan penurunan kandungan melanin antara kepekatan 0.025-0.1% yang lebih baik daripada asid kojic. Kandungan melanin untuk kedua-dua pecahan metanol 40% dan 100% adalah masing-masing 64.95 ± 1.87% dan 71.91 ± 1.18% dan secara signifikan menghalang aktiviti tirosinase dengan kebergantungan kesan pecahan metanol *L. pumila* kepada dos terhadap sel melanoma B16F1. Walau bagaimanapun, pecahan metanol 40% mempamerkan perencatan tertinggi sehingga 73.96 ± 1.58% bagi aktiviti tirosinase. Secara keseluruhannya, kajian ini menunjukkan bahawa pecahan metanol *L. pumila* mempunyai potensi yang baik sebagai sumber perencat tirosinase.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

TPC	-	Total phenolic content
MeOH	-	Methanol
SPE	-	Solid phase Extraction
MTT	-	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium
α-MSH	-	α- Melanocytes-stimulating hormone
MITF	-	Microphtalmia-associated transcription factor
POMC	-	Proopiomelanocortin
MC1R	-	Melanocortin-1 receptor
ATP	-	Adenosine triphosphate
cAMP	-	Cyclic adenosine mono-phosphate
$_{L}$-dopa	-	L-3,4-Dihydroxyphenylalanine
TRP	-	Tyrosinase-related protein
DCT	-	Dopachrome tautomerase
DHICA	-	Dihydroxyindole- carboxylic acid
DHI	-	Dihydroxyindole
DMSO	-	Dimetyl sulphoxide
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylene diamine tetraacetate
UV	-	Ultraviolet
IBMX	-	Isobutyrylxanthine
PKC	-	Protein kinase
ROS	-	Reactive oxygen species
PBS	-	Potassium phosphate buffer saline

CHAPTER 1

INTRODUCTION

1.1 Research background

In recent years, dermatologist and cosmetologist are formulating cosmetics with whitening effect. It has such a big demand especially in the Asian region where people are obsessed with fair and radiant skin. In early 1990s, cosmetic companies had begun to use the term cosmeceuticals to describe skin care products that are claimed to have therapeutic benefits through their bioactive ingredient and exert tangible effects on users. In year 2000, the term cosmeceutic was extended to cover 'the functional cosmetic products' that go above and beyond their intended function by offering an additional therapeutic benefit (Park, 2009).

High awareness among consumers in the past few years has brought about significant development in sales and technology of global cosmetics and toiletries. It may trigger significant growth across markets segments and geographies. The high demands create large opportunities which encourage the emergence of new inventors in this field due to the fast growth of research and development annually (Wang *et al*., 2006)

One of the primary causes to speed up the market growth in this field is the changing of production unit to be more cost effective as observed to be widely practiced within Asia such as in India and China. Besides, the applications of online selling by retailers can help to speed up the growth of the market. Increasing awareness among consumers towards natural products will force the cosmetics and toiletries manufacturers to go with the flow in order to attract consumers (Transparency Market Research, 2013).

According to Transparency Market Research, development of global cosmetics and toiletries market is affected by economic environment and by the year of 2015 it is estimated to reach USD19.2 billion. However, gaining trust from the consumers is quite challenging but fast development of domestic market can cover up the economic crisis in this field. The market is still at the beginning stage and must be concrete enough for future potential (Transparency Market Research, 2013).

Currently, male products can beat female products and it has experienced quite an important growth in the market. The addition can contribute approximately $4 billion to the global value size which is 27 billion by 2014 (Carrie, 2010).

High demands of natural products have increased gradually in the European and North American market but the industry is facing high cost challenge. Natural products give high confidence in terms of consumers' health, concerns on environmental, and the carcinogenic nature of the synthesis source. These issues will give major contribution in speeding up the growth of natural products (Transparency Market Research, 2013).

The demand for cosmetics is increasing due to the obsession of having fairer and radiant skin especially in the Asian region. Recently, many whitening products on the shelves have been recalled due to the use of restricted chemical compounds

(Eisah, 2013). The main consideration of those called in products is on the safety and effectiveness of the product claims.

In Malaysia it is a trend to have a radiant and fair skin complexion. Most of the products in the local market claim to be effective in lightening the skin. The consumers are also inclined to select natural products over cosmeceutical blending that uses toxic chemicals. Fruits, vegetables and products derived from animals are great sources of antioxidant, rich in substances that can improve human complexion, moisturize skin, attenuate fine lines and wrinkles, and give elasticity to the skin (Ferris, 2007). There is a number of natural ingredients that can be used to remove dark spots and whiten skin (Briganti *et al*., 2003) and just as many reasons to try them.

There has been a discussion on how to determine the reduction in melanin based on the efficacy of whitening products without human study. Animal tests are also not permitted in most countries. As a result, *in vitro* study of cell culture was identified to be a good alternative to assess the tolerance and efficacy of products to ensure maximum safety (Donald *et al*., 2006).

Skin pigmentation processes involve the *de novo* synthesis of melanin in melanocytes and transfer of the synthesized melanin packed in melanosome to neighboring keranotycytes, which eventually turns the skin color into a darker tone (Hearing, 2005 and Seiberg *et al*., 2000). Melanosome contains three types of enzyme, tyrosinase, TRP1, DCT (TRP2) (Kobayashi *et al*., 2007). Tyrosinase is a rate limiting enzyme involved in melanin synthesis that hydroxylates tyrosinase, a kind of phenylalanine, to L-3, 4-dihydroxyphenylalanine (L-DOPA) and oxidizes L-DOPA to DOPA quinine (Kim *et al*., 2006). Excessive accumulation of DOPA quinine generated from hydroxylation and oxidation of tyrosinase forms DOPA chrome, which conditionally exhausted systeine, resulted in accumulation of black and brownish pigment called eumelanin. Another type of melanin, pheomelanin is produced through formation of 3 of 5 systeinyl DOPA on condition of existence of

systeine (Kim *et al*., 2006; Miyamura *et al*., 2007 and Yamaguchi *et al*., 2007). These three enzymes determine the types of melanin to eumelanin or pheomelanin (Hearing. 2005). Accordingly, our skin color can be determined by the ratio between the types of melanin, the amount of each type of melanin and the extent of transferring melanosome to keratonocytes (Hamesath *et al*., 1998).

There are several factors that can modulate the melanin biosynthesis. Melanin biosynthesis can be reduced by avoiding UV exposure, by inhibition of melanocytes metabolism and proliferation (Kim and Uyama, 2005), and by inhibition of tyrosinase or by removal of melanin corneal ablation. Inhibition of tyrosinase oxidation catalyzed by tyrosinase is one of the factors to avoid melanin production (Slominski *et al*., 2004).

Recently, the demand for natural products that inhibit or prevent skin pigmentation is increasing all over the world. A variety of natural or synthetics substances are currently utilized as ingredients of preparations designed to control hyperpigmentation, but none of these have been proven to be completely satisfactory, either due to limited efficacy or owing to safety concern. For instance, hydroquinone, which has been used widely and until recently was considered the standard depigmenting agent, has now been banned for cosmeticeutical uses in Europe and some Asian countries, and is available only by prescriptions. Kojic acid, also used in skin lightening, is based on blocking the activity of tyrosinase. However, there are reports showing skin sensitization, including dermatitis and cytotoxicity, which have resulted in some countries like Japan, Switzerland and Korea, discontinuity of its use (Serra-Baldrich et. al., 1998). Recently, skin lightening is also a cause of mercury toxicity. The features of mercury toxicity, also known as the ‘hatters disease’, as immortalized in *Alice in Wonderland* by Lewis Carroll, consists of psychiatric (disturbance or recent memory, impairment of intellectual function, inattention and depression) and neurological (irritability, memory loss and neuropathies) problems (Dadzie and Petit, 2009).

Thus a study was undertaken to investigate if *L. pumila* possesses any tyrosinase inhibition effects with a view of its possible use as a treatment for hyperpigmentation and its use as a skin whitening agent in cosmetics.

1.2 Problem statement

Over the last few decades, much effort had been exerted to search for tyrosinase inhibitors. A large number of compounds, both from natural products and synthetic compounds were assayed on tyrosinase, but only a limited number of them could fulfill the requirement of efficacy and safety. Common examples of extracts used as whitening is green tea, mulberry, and so on. However, the commercial value of each potential plant is still in a question for its prospects. High efficacy and safety still remain as the main considerations for human use and every active compound identified needs further investigation especially in humans. For those to be applied in food products, it is also important that they do not cause significant negative impact on the sensory properties. Therefore, many plants have been investigated to determine their potential for application as cosmetic agents.

Traditional herbal plants like *L. pumila* are great alternatives since commercial tyrosinase inhibitors such as hydroquinone and kojic acid could bring side effects to human because of their toxicity (Moussy *et al*., 2004 and Briganti *et al*., 2003). Previous studies have shown that *L. pumila* contains many phytochemicals that have been identified to exhibit beneficial properties such as antioxidant, antimicrobial and anti-estrogenic diseases (Chua *et al*., 2012). It has been reported that antioxidant may reduce hyperpigmentation and support skin health (Ma *et al*., 2001). However, the inhibition activity of tyrosinase by *L. pumila* has not been established.

1.3 Hypothesis

The central hypothesis of this study is that the methanol fractions of *L. pumila* exhibit anti- tyrosinase properties.

1.4 Objective of the study

The aim of this study is to establish the tyrosinase inhibition in melanogenesis by *L. pumila* methanol fractions using B16F1 murine melanoma cells.

1.5 Scopes of the study

This study consists of three main scopes. They are outlined as follows:

1. To investigate the effect of *L. pumila* methanol fractions on the cells viability of B16F1 murine melanoma cells.

2. To determine the concentration effect of *L. pumila* methanol fractions on the formation of melanin content in B16F1 melanoma cells.

3. To investigate the concentration effect of *L. pumila* methanol fractions on the inhibition of tyrosinase activity using enzymatic assay.

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